EXTRUSION AS A MECHANISM OF HOST IMMUNE RESPONSE EVASION IN A *CHLAMYDIA TRACHOMATIS* MURINE INFECTION MODEL

By

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Abstract: *Chlamydia trachomatis* is an obligate intracellular organism that is the leading cause of preventable blindness and sexually transmitted bacterial infections. *C. trachomatis* exhibits a biphasic developmental cycle involving infectious elementary bodies (EB) and non-infectious, replicative reticulate bodies (RBs). At the end of its developmental cycle, EBs disseminate to neighboring cells either via host cell lysis or a novel mechanism of exit, called extrusion. It has been hypothesized that extrusions serve as a means of immune response evasion due to enclosure within host membrane. In addition, extrusions filled with multiple EBs may serve as a mode for high dose delivery of infectious organisms to tissues, rather than individual EBs from lysed host cells. Herein, female mice were intra-vaginally infected with either a *C. trachomatis* serovar L2 wild type strain or a mutant strain containing a silenced CT228 gene, which produces significantly more extrusions *in vitro*, relative to the wild type. All mice were characterized for, i.) time course of infection, ii.) systemic and mucosal immune response to infection, iii.) degree of reproductive tissue damage following clearance of infection, and iv.) recruitment of different immune cell types to reproductive tracts. In comparison to the wild-type strain, mice infected with the mutant strain revealed an increase in the time needed to clear infection, a reduction in the systemic anti-*Chlamydia* antibodies, and a decrease in mucous production. However, there were no significant differences amongst the concentrations of immune cells recruited to the reproductive tracts. These data may suggest that EB antigen within extrusions dampen recognition by the immune system. Therefore, further research is warranted to quantitate cytokine concentrations, to examine antigen presentation, and to examine the development of protective immunity.

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CHAPTER I

BACKGROUND AND INTRODUCTION

Chlamydia trachomatis is one of the most common bacterial sexually transmitted diseases worldwide and, even if treated or upon recurrence, it can lead to a myriad of complications including infertility, pelvic inflammatory disease, loss of vision, and ectopic pregnancies (Haggerty et al., 2010). There has been substantial evidence that associates chlamydial genital mucosa infection to an increased risk for ovarian and cervical cancers as well (Das, 2018). The species consists of two separate biovars that are further categorized into 15 serovars based on variable regions of the surface antigens present on the major outer membrane protein encoded by ompA, MOMP (Byrne, 2010). These serovars present as different types of disease: Serovars A-C are responsible for trachoma, the most preventable cause of vision loss in underdeveloped countries, serovars D-K are responsible for disease of the genital mucosa and are the most common form of sexually transmitted infection (STI) reported, and serovars L1-L3 are responsible for lymphogranuloma venererum, an invasive STI (Schachter, 1999). In the US alone, there are over 1 million cases that are reported each year within the past decade with approximately 100 million genital infections occurring annually worldwide (World Health Organization, 2008). However, due to the occurrence of asymptomatic infections

that are predominately presented in women, the predicted number of cases may be under-reported.

Chlamydia spp. are obligate intracellular bacteria known to possess a unique biphasic developmental cycle that involves the transition between two distinct forms: the intracellular reticulate body (RB), which is the non-infectious, metabolically active, replicative form (RB) and the extracellular elementary body (EB), which is the infectious, non-metabolically active, non-replicative form (Moulder, 1991) (Fig. 1). Initiation of infection occurs once an EB binds to the surface of an epithelial cell followed by internalization of the EB. During internalization, the EB resides in a host-derived membrane vesicle termed the inclusion. The inclusion provides a safe environment for the bacteria to thrive and serves as the interface between the bacteria and the host cell, which allows acquisition of nutrients, lipids, and aid in trafficking (Bastidas et al., 2013). It is within the inclusion that the transition of EBs to RBs occurs whereby, during the first 20hrs, the RBs replicate via a polarized budding process (Abdelrahman et al., 2016). After this time point, the population begins an asynchronous transition back into the EB form. Depending on the species or the cell lines used for growth of the organisms *in vitro*, the EBs escape from the infected host cell after 48 to 72hrs either via lysis or another mechanism of exit termed extrusion (Hybiske and Stephens, 2007).

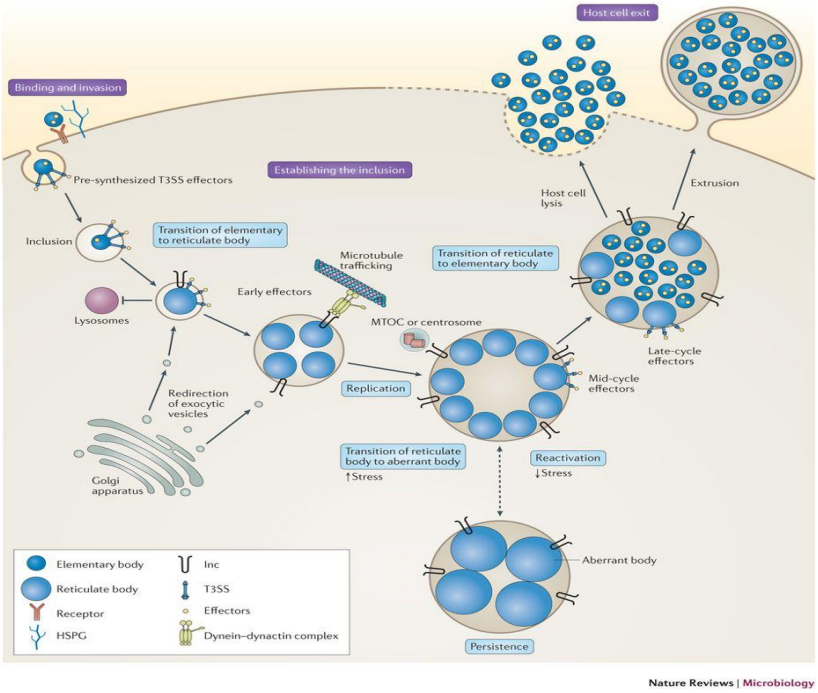


Figure 1. Chlamydial Developmental Cycle (Elwell et al., 2016)

Due to the obligate intracellular lifestyle coupled with the unique biphasic developmental cycle, vaccine development and genetic manipulation have been historically underwhelming. Many of the vaccine strategies investigated since the early 1960's mainly targeted a component of serovars A-C, which are responsible for trachoma. Trachoma is the leading cause of bacterial induced vision loss in under-developed countries, and even with treatment, irreversible scarring resulting in blindness can occur (Burton and Mabey, 2009). The availability of antibiotics required to clear this infection is scarce in many of these regions which is further compounded by poor hygienic living conditions thus prompting the demand for an increase in chlamydial vaccine-based research. Since discoveries made in the 1960's, there have been numerous potential vaccine candidates against both *C. muridarum* and *C. trachomatis* strains (Schautteet et al., 2011). This includes the development of a vaccine in Phase 1 that

induces immunity against trachoma in nonhuman primates (Kari et al., 2011). In the past decade, there have been significant advancements in chlamydial genetics across all species. These include the development of a conditional expression vector that utilizes a tetracycline-inducible system (Bauler and Hackstadt, 2014), the development of vectors that confer blasticidin and chloramphenicol resistance (Ding et al., 2013; Xu et al., 2013), the ability to perform forward and reverse genetic screens due to the use of chemical mutagenesis (Nguyen and Valdivia, 2012), and the ability to employ targeted mutagenesis techniques using TargeTron, a group II intron-based gene knockout system (Johnson and Fisher, 2013). Such advancements have led to the discovery and elucidation of virulence factors, polymorphic membrane proteins, and phosphatases.

During the developmental cycle of *Chlamydia spp.*, the inclusion membrane serves as the interface between the bacteria and the host cell that undergoes modification for functions such as trafficking to the MTOC (Clausen et al., 1997) and host cell lipid acquisition (Hackstadt et al. 1996). The membrane of the inclusion is decorated with different Type III secreted proteins termed inclusion membrane proteins, or Incs, that play critical roles in autophagy avoidance, promotion of nutrient acquisition, innate signaling, fusion of membrane, etc (Moore and Ouellette, 2014). The exact number of Incs varies per chlamydial species, but to date, *C. trachomatis* is predicted to contain 36-59 of these proteins (Shaw et al., 2000; Lutter et al., 2012). Few have been characterized using *in vitro* methods over the past decade due to the lack of chlamydial genetic advancements (See Table 1), and regarding the few Incs in which function has been elucidated, *in vivo* studies involving animal infection models have been nonexistent.

Table 1. List of characterized Incs

Inc	Function	Cited Source
IncA (CT119)	Homotypic fusion of inclusions; Encodes SNARE-like motifs	(Hackstadt et al., 1999)
IncB (CT232)	Contains Src kinases; aids in intracellular growth and migration of inclusion	(Mital et al., 2010)
IncD (CT115)	Recruits a protein responsible in transfer of lipids, known as ceramide endoplasmic reticulum transferase (CERT)	(Agaisse and Derre, 2014)
IncG (CT118)	Enhances host cell viability via recruitment of adaptor protein, 14-3-3β	(Scidmore and Hackstadt, 2001)
IncV (CT005)	Aids in attachment of inclusion to the endoplasmic reticulum (ER) via the interaction with ER integral membrane VAP	(Stanhope et al., 2017)
CT229	Interacts with GTPase Rab4 to regulate endosomal trafficking	(Rzomp et al., 2006)
CT850	Promotes migration of inclusion to the MTOC via the interaction with dynein	(Mital et al., 2015)

CT813	Promotes migration of inclusion to Golgi mini-stacks via the binding to ARF1 and ARF4	(Wesolowski et al., 2017)
CT101 (MrcA)	Regulates host cell exit via extrusion; utilized for management of calcium via the interaction with host inositol tri-phosphate (IP_3)	(Nguyen et al., 2018)
CT228	Regulates host cell exit via extrusion; Recruits myosin phosphatase (MYPT1) to inclusion membrane	(Lutter et al., 2013)

Since the early 1980's, extensive knowledge regarding pathogenicity, vaccine candidates, immune response, and fertility has been acquired from murine infection models infected with either *Chlamydia muridarum* or *Chlamydia trachomatis* strains (Perry et al., 1997; Morrison and Caldwell, 2002; Morrison et al., 2011; De Clercq et al., 2013). Murine models are the most commonly used animal models in chlamydial research, and it is important to note the differences in these murine models infected with either *C. muridarum* or *C. trachomatis*: Genital inoculation with *C. muridarum* typically leads to more virulent, tissue damaging sequelae that closely mimics human histopathology in comparison to genital inoculation with the human serovars (Lyons et al., 2005). *C. muridarum* infection also naturally ascends from the tissues of the lower

genital tract to establish itself in the upper genital tract tissues (Naglak et al., 2016). Further discussion of the immune response to genital chlamydial infection is detailed in Chapter 3.

Comparative studies involving the utilization of different techniques for the infection of different mouse strains with multiple chlamydial strains have been examined, and all of these studies have utilized wild-type strains of *Chlamydia* only. Although there has been substantial *in vivo* research over the past 70 years involving the identification of a possible vaccine candidate that has predominantly favored the targeting of *C. trachomatis* and *C. muridarum* via the delivery of the major outer membrane protein (MOMP) within mice (Phillips et al., 2019), other features of these bacteria such as the mechanism of extrusion formation during host cell exit and the effects of these extrusions on the host immune response has been lacking.

The process of extrusion as a method of host cell exit is unique to *Chlamydia spp.* and has been hypothesized to be involved in host immune response evasion, dissemination of infectious progeny to different tissues, and the spread of infectious progeny from cell-to-cell (Hybiske and Stephens, 2007). The presence of extrusions was recognized *in vitro* several decades ago (Todd and Caldwell, 1985). However, they have only recently been shown to occur *in vivo* using a murine infection model (Shaw et al., 2017). In this study, extrusions were successfully harvested and imaged via microscopy. The availability of the tools gained from this study has facilitated further examination of this phenomenon, taken together with new genetic systems enabling the assessment of mutant strains with disrupted regulation of extrusion formation *in vivo*. Data suggest that the presence of extrusions may lead to an altered immune response suggestive of evasion.

Purpose and Aims of Study

In the field of chlamydial pathogenesis, there are currently no published data that encompass infection of an animal model with *Chlamydia trachomatis* mutant strains with a known phenotype that have been fully characterized. There is also a lack of exploratory studies into the possibility that inclusion extrusion provides a mechanism for immune response evasion. The overall objectives of this study are to utilize *in vivo* methods to gain a more profound understanding of chlamydial urogenital infection and the host immune response using *C. trachomatis* mutant strains that function in the regulation of extrusion formation *in vitro,* and to determine the potential effects of extrusions *in vivo* and their role in immune response evasion. **Our central hypothesis is that *Chlamydia* host cell exit via inclusion extrusion enables evasion of the host immune response.** In order to test our central hypothesis, we propose two aims:

Aim 1: Utilize a murine infection model to examine and compare the physiological and immunological effects of a murine L2-wild type infection versus a mutant L2 infection in which the mutated gene product is known to have a role in the regulation of extrusion formation *in vitro.*

Aim 2: Examine and compare the recruitment of different immune cells to whole reproductive tracts of a murine L2-wild type infection versus a mutant-L2 infection that was utilized in Aim 1.

CHAPTER II

GENETIC INACTIVATION OF *CHLAMYDIA TRACHOMATIS* INCLUSION MEMBRANE PROTEIN CT228 ALTERS MYPT1 RECRUITMENT, EXTRUSION PRODUCTION, AND MURINE INFECTION

It has been hypothesized that the extrusion exit mechanism may either, (1) maximize the dissemination of vesicles that are abundant in *Chlamydia* instead of maximizing dissemination of individual EBs to neighboring host cells or, (2) provide a means to evade or subvert the host immune response temporarily via the enclosure of infectious progeny within a portion of host cell membrane. *In vitro*, the occurrence of extrusions has been documented for many years, but only recently was an extensive *in vitro* analysis of their morphology and function performed. This particular study showed that macrophages are capable of phagocytosing extrusions and promoting chlamydial survival (Zuck et al., 2017). There has also been a study that identified protein interactions that play a role in host cell egress allowing us to examine host immune response evasion in more detail (Lutter et al., 2013). Recently, in 2017, *in vivo* documentation of extrusion occurrence was finally obtained across multiple wild type serovars, in which extrusions were successfully harvested from a murine infection model and imaged via microscopy (Shaw et al. 2017). However, there remains a need to gain a

deeper understanding as to how and why extrusions serve as an alternative mechanism of exit as well as their effects on the host immune response.

Since extrusions are now capable of being harvested, imaged, and quantified *in vitro*, it is apparent that selection of mutants expressing altered chlamydial proteins known to regulate the formation of extrusions *in vitro* for the use in an animal infection model is a significant step. Due to genetic advancements in the field, identifying target proteins that regulate this process is now possible. *In vitro* studies demonstrated that *C. trachomatis* inclusion membrane protein CT228 negatively regulates extrusion formation via the complex interaction between this Inc and host myosin phosphatase target subunit (MYPT1) (Fig. 2). *Chlamydia* exit via extrusion or lysis depends on the phosphorylation state of myosin light chain 2 (MLC2): dephosphorylation of MLC2 enhances the process of lysis, while phosphorylation of MLC2 promotes extrusion. Recruitment of MYPT1 by CT228 to the inclusion alters the phosphorylation state of MLC2 thereby regulating exit from the host cell. (Lutter et al. 2013).

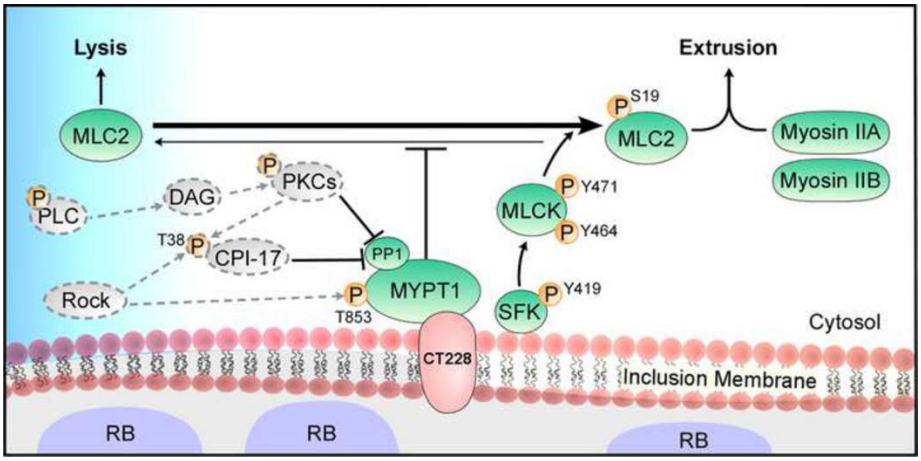


Figure 2. Representation of myosin phosphatase pathway in *C. trachomatis* in relation to CT228 (Lutter et al. 2013)

For this study, we utilized a mutant generated by TargeTron, a gene knockout system, which consists of a targeted chromosomal mutation in *C. trachomatis* serovar L2 inclusion membrane protein CT228 (L2-ΔCT228). We examined immunological and phenotypical differences in response to infection with wild type *C. trachomatis* serovar L2 versus L2-ΔCT228 by monitoring the course of infection, examining the histology of the whole murine reproductive tracts, and obtaining anti-*Chlamydia* antibody titers in sera and vaginal washes.

Hypothesis and predictions

We hypothesized that there would be immunological and pathological differences between the wild type *C. trachomatis* infection and L2-ΔCT228 infection. A prior *in vitro* study revealed that L2-ΔCT228 produced more extrusions *in vitro* (Lutter et al. 2013). If immune response evasion was observed, we could **predict** that, in comparison to a wild type infection, the mutant strain would produce a prolonged infection, a decrease in damage to the reproductive tract, and a decrease in antibody concentration obtained from sera and vaginal washes.

Methods

Intravaginal Infection of Mice

Female inbred C3H/Hej mice were purchased from Jackson Laboratories (Bar Harbor, Maine) at six weeks old and were acclimated for one week prior to any experimentation. All mice were subcutaneously injected with Depo Provera formulation of 2.5mg of medroxyprogesterone acetate (Upjohn, Kalamazoo, MI) at 7 and 3 days before infection

to synchronize estrus. On the day of infection, mice were infected intravaginally with 1 million *C. trachomatis* L2-wild type EBs or L2-ΔCT228 EBs in 5ul sucrose-phosphate-glutamate (SPG). The course of infection along with the histopathology (n = 5/group) or immune response (n = 8-9/group) were monitored.

Quantification of Recoverable IFUs from the Cervicovaginal Tract

Cervicovaginal tracts were swabbed (Puritan Diagnostics, HydraFlock 6" 15 cm swabs; Guilford, ME) at days 7, 14, 21, 28, and 42 post infection (p.i). As adopted from previous studies, each swab was added to microcentrifuge tubes containing 600ul of SPG and two glass beads and placed on ice. EBs were liberated from each swab via vortexing and used in serial dilution and inoculation of confluent HeLa cell monolayers in 96 well plates (CellTreat Scientific, Pepperell MA). EB entry was promoted via centrifugation at 700 x g for 1 hour. The cells were then incubated at 37°C with 5% CO_2 for 30 min. before washing to remove any extracellular EBs. After washing, these cells were incubated in RPMI 1640 + 5% FBS + gentamycin (10ug/mL) for 24 hrs. at 37°C with 5% CO_2. The cells were fixed with methanol, stained with anti-MOMP (courtesy of Dr. Harland Caldwell) followed by staining with anti-mouse DyLight 488 (Jackson ImmunoResearch). A Leica MI6000B fluorescent microscope with a 20x objective was used to count a total of twenty fields of view in order to obtain a calculation of total recoverable IFUs per mouse.

Quantification of Mucosal and Systemic Antibody Response

Vaginal lavages and sera were collected at day 31 p.i. for ELISA to measure both systemic and mucosal immune responses to chlamydia infection. Vaginal lavages were collected by washing the vaginal vault with 60 ul 0.5% BSA-PBS twice, and each of the samples collected was stored at -20°C until ELISA was performed. 1 ug of formalin fixed EBs (serovar L2) was used to coat each well of 96-well polystyrene plates (Immulon 2HB; Thermo, Milford, MA) in 100 ul TBS (50 mM Tris buffer pH 7.5, 0.15 M NaCl) and were incubated at 4°C overnight. After this incubation period, wells were washed to remove any unbound EBs and then blocked with 200ul 2% BSA in 0.012 M Tris pH 7.4, 0.14 M NaCl, 3.0 mM KCl, 0.05% Tween 20 for 90 minutes at 37°C. Serial dilutions of vaginal washes and sera were added to washed plates and incubated at 37°C for 90 min. Alkaline anti-mouse IgG and IgGA antibodies (vaginal lavage samples) with anti-mouse IgG2a (sera samples) (Southern Biotech Associates, Burningham, AL) were used to measure *Chlamydia*-specific antibody titers. Absorbance was read at 405nm (Biotech Synergy, Winooski, VT) after the addition of the substrate, P-nitrophenyl phosphate (pNPP) to determine relative concentrations of antibody titers. The highest sample dilution that displayed an absorbance more than or equal to 3X the absorbance of the concentrated uninfected (sham) was considered positive.

Reproductive Tract Histology

On day 23 and day 64 p.i. the whole reproductive tracts of mice were excised and immersion fixed in 10% buffered neutral formalin with the purpose of scoring inflammatory damage. After the process of fixation, these tracts were processed in whole

and embedded *en bloc* into paraffin, cut into sections at 4 µm, and stained with eosin and hematoxylin (H&E). Each section was examined by an American College of Veterinary Pathologists (AVCP) certified veterinary pathologist and scored with the respective designations: 0=normal, 1=minimal change, 2=mild change, 3=moderate change, and 4=severe change. Uterine stratum and luminal compactum inflammation, hydrosalpinx, periglandular mucinous change, and overall impression were all the parameters that were scored using the above designations. Olympus CellSens software linked to an Olympus DP70 microscope camera were used to take calibrated measurements of endometrial luminal epithelial height morphometric analyses. A total of six locations (distal, proximal, and mid-locations of both uterine horns) of each whole reproductive tract was evaluated morphometrically and regionally. Each of the locations was determined to be free of tissue curves and bends as well as uniform.

Statistics

The total extrusion numbers between L2-wild type and L2-ΔCT228 as well as the mean +/- SE growth (IFUs) were compared using the student's unpaired two-tailed t-test with equal variance. Log IFU data was analyzed across four time points following infection using one-way ANOVA repeated measures. Both mucosal (IgA and IgG) and systemic (IgG2a) mean +/- SE antibody titers were analyzed using student's unpaired two-tailed t-test. The same test was used on the histopathology clinical scores between both types of infection. Prism 5.0 was utilized to obtain statistical analyses, and for all analyses, data were considered to be significant at $p<0.05$.

Results

From this study, it was determined that infection with the mutant strain produced alterations in the duration of chlamydial infection, in the mucinous changes in the reproductive tracts, and in the concentration of antibodies present in the mucosa and sera. The quantitated recoverable log IFUs, as shown in Fig. 3, revealed a statistically significant effect of time on the clearance of L2-wild type ($p=0.006$) that was absent in response to L2-ΔCT228 ($p=0.371$). Only 3/9 L2-ΔCT228 infected mice were clear by day 28 in comparison to the 6/8 L2-wild type infected mice.

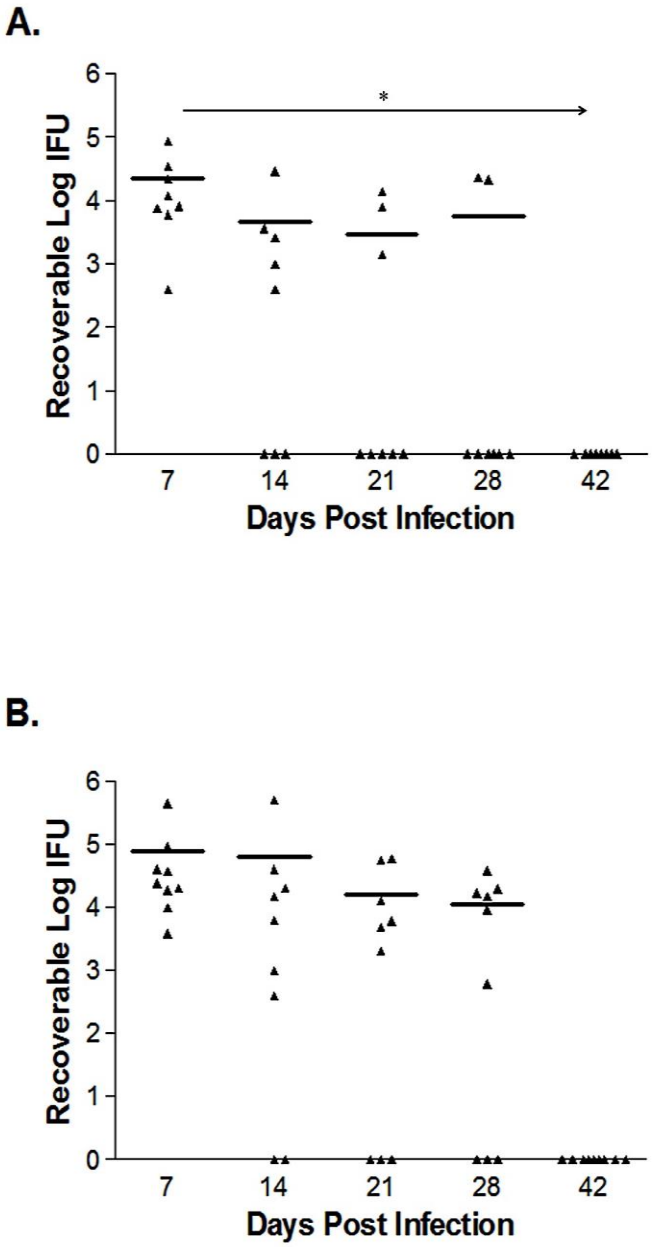


Figure 3. Recoverable IFUs shed by mice infected with *C. trachomatis* L2-wild type and L2- CT228. Female C3H/HeJ mice were intravaginally infected with 1×10^6 EBs of either L2-wild type or L2- CT228. Recoverable IFUs were obtained by swabbing vaginal tracts and enumerating on HeLa cell monolayers. Recoverable IFU data are expressed for **(A)** L2-wild type ($n = 8$) and **(B)** L2- CT228 ($n = 9$) on a logarithmic scale from Day 7 to 42 post-infection. Effect of time ($^*p = 0.006$) was observed in mice infected with L2-wild type (repeated measures one-way ANOVA). For day 28, 6/8 L2-wild type infected mice were clear compared to 3/9 L2- CT228. Triangles represent individual mice, bars represent mean of group for each time point.

At day 31 p.i., L2-ΔCT228 infected mice produced significantly lower systemic anti-*Chlamydia* IgG2a titers relative to L2-wild type infected mice, and there were negligible mucosal IgG titers in both groups at Day 31 which is expected in response to infection with serovar L2 as presented in Shaw et al. 2018. Mean mucosal IgA titers were very low. However, there was a slight decrease observed in the mice infected with L2-ΔCT228 (Fig. 4).

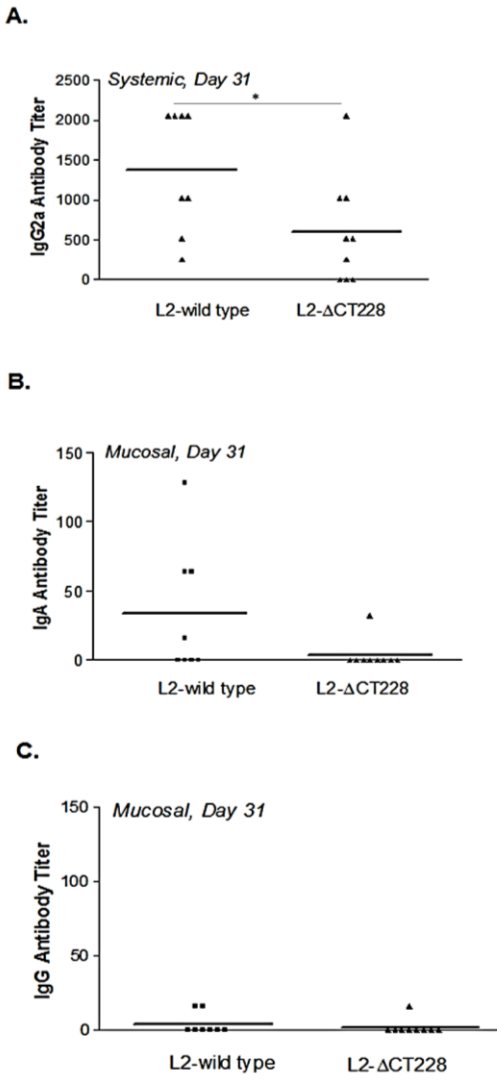


Figure 4. Systemic and mucosal antibody titers following infection with *C.*

***trachomatis* L2-wild type and L2- CT228. (A)** Sera were collected from mice 31 days post-infection with L2-wild type (n = 8) or L2- CT228 (n = 9) and assayed for the presence of anti-chlamydial IgG2a. **(B,C)** Vaginal lavages were collected 31 days post-infection and assayed for the presence of anti-chlamydial IgA and IgG. Antibody titers are expressed for individual mice as the highest dilution tested that produced >3-fold the absorbance as control/uninfected mice. Bars represent mean antibody titer per group. *p = 0.0412, unpaired two-tailed Student t-test.

Finally, there were slight decreases in mucinous changes and inflammation following infection with L2-ΔCT228 than with L2-wild type according to the histological analyses of whole murine reproductive tissue at Days 23 and 64 post-infection (Fig. 5 and Table 2). The decreases in mucinous changes and inflammation may or may not be of any biological consequence due to the low reliance and pathological relevance of the clinical scoring parameters that are utilized across different chlamydial studies. *C. trachomatis* serovar L2 is also known to elicit marginal histopathological changes in a murine model that are not considered significant (Shaw et al., 2017).

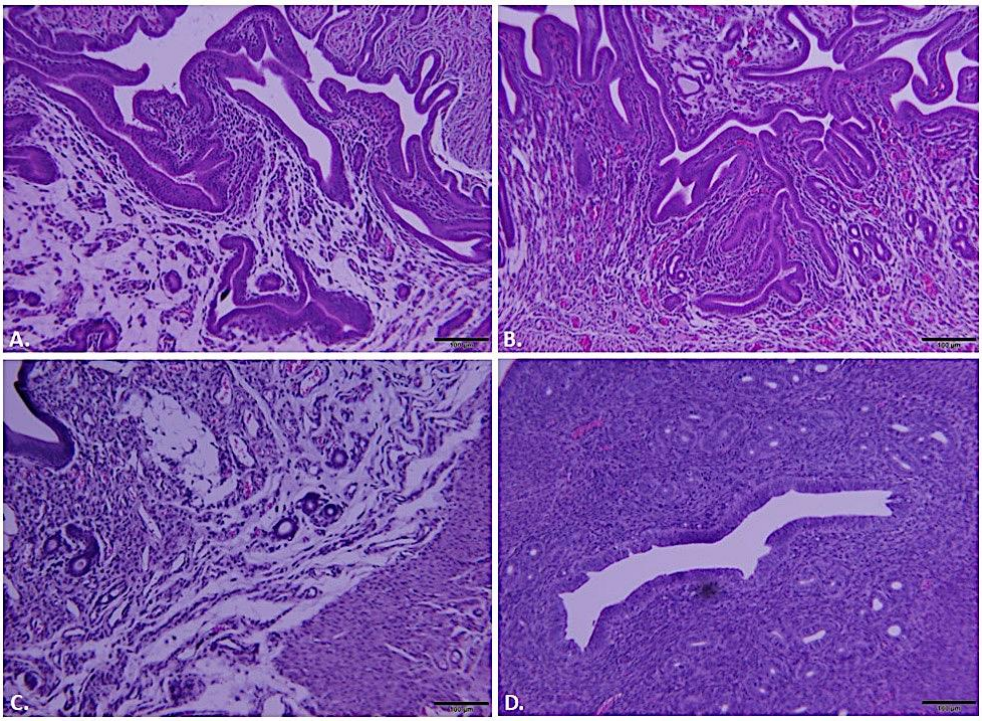


Figure 5. Histopathological assessment of reproductive tracts post-infection. Mice (*n*

= 5 per group) were euthanized at 23 and 64 days post-infection (dpi) and entire reproductive tracts were removed and formalin fixed for histology. Representative images of H&E stained sections of uterine tissue were captured using an Olympus DP70 for mice infected with **(A)** *C. trachomatis* L2-wild type, 23 dpi, **(B)** *C. trachomatis* L2-CT228, 23 dpi, **(C)** *C. trachomatis* L2-wild type, 64 dpi, **(D)** *C. trachomatis* L2- CT228, 64 dpi. See **Table 2** for pathological clinical scoring of reproductive tracts at 64 dpi.

Table 2. Pathological scoring of murine reproductive tracts.

Infectious agent	Overall impression	Mucinous change	Hydrosalpinx	Uterine tubal dilation	Uterine luminal PMN	Representative images
L2-Wild type	1, 0, 1, 0, 3	2, 1, 2, 1, 1	0	1, 1, 0, 2, 3	0	Fig 6A,6C
L2-ΔCT228	2, 0, 0, 0, 0	0, 1, 0, 1, 0*	0	2, 0, 0, 1, 0	0	Fig 6B, 6D

*Histological sections of reproductive tracts (n = 5/group at 64 dpi) were examined and scored by an ACVP certified veterinary pathologist via light microscopy with the following numerical designations: 0, normal; 1, minimal change; 2, mild change; 3, moderate change; 4 severe change (mean ± SE). *p = 0.02 (L2-wild type vs. L2- CT228).*

Discussion

Based on *in vitro* research, L2-ΔCT228 produced significantly more extrusions than L2-wild type in a HeLa tissue cell culture line. Our central hypothesis is that *Chlamydia* host cell exit via inclusion extrusion enables evasion of the host immune response *in vivo.* EB enclosure within increased numbers of extrusions that are enveloped by portions of the host cell membrane may alter the ability of the host immune response to recognize EB antigens. Such a phenomenon may support the significant decrease in clearance at day 28 p.i. of mice intravaginally infected with L2-ΔCT228. The data above reveals that only 3/9 L2-ΔCT228 infected mice cleared by day 28 versus the 6/8 L2-wild type infected mice (Figure 3). In addition, L2-ΔCT228 infected mice produced

significantly lower systemic anti-*Chlamydia* IgG2a titers at day 31 p.i. (Figure 4A) along with negligible mucosal IgA and IgG titers that were also present at negligible levels in the wild type strain (Figure 4B,C). The histological analyses listed above also revealed a significant but minor decrease in the production of mucous within sections of murine uterine tissue at day 23 and day 64 p.i. with L2-ΔCT228 in comparison to L2-wild type (Table 2.). In comparison to other genital serovars, serovar L2 elicits minimal histopathological changes, and any interpretations of these changes are restricted due to the low reliance and restricted parameters of the clinical scoring system. From previous studies, the clinical scores of mucinous changes in L2-wild type infected mice revealed an overlapping but lower numerical range despite the prevalence of higher recoverable IFUs (Shaw et al., 2017) than what was revealed in this study.

Overall, these *in vivo* findings reveal a delay in clearance following intravaginal infection with L2-ΔCT228, along with an associated decrease in anti-*Chlamydia* humoral responses. We assume that these differences along with the differences in EB release via lysis or extrusion may affect the ability of host immune response to clear infection. This may be due to the possible alteration in the degree of antigen presentation and recognition via dendritic cells and macrophages. Disruption of CT228 and the loss of MYPT1 recruitment, as a result, have an effect on the longevity of infection *in vivo.* This may be in relation to the degree of extrusion-mediated host cell exit.

Future directions would include the elucidation of mechanisms mediated by L2-ΔCT228 that are involved in host immune response evasion/alteration. This would involve the characterization and enumeration of immune cells recruited to murine reproductive tracts that will play roles in antigen presentation, initial clearance,

development of protective immunity, interleukin and IFN-y production, and the onset/occurrence of cytotoxic events. The development of a CT228 mutant in other serovars that more closely mimic the histopathology observed in human disease is crucial. The investigation on the effects of the functions and localization of other Incs as a result of CT228 inactivation would be of interest as well.

CHAPTER III

POPULATIONS OF IMMUNE CELLS RECRUITED TO WHOLE MURINE REPRODUCTIVE TRACTS BY *CHLAMYDIA TRACHOMATIS*

In order to gain a thorough understanding of the immune response to genital infections, animal models are implemented, such as murine, guinea pig, porcine, macaque, and non-human primate models. The murine model is most commonly used for chlamydial research with many advantages: lower costs, smaller size, and availability of transgenic strains as well as mouse-specific reagents. In comparison to genital infection with *C. muridarum*, which is frequently used for genital infection studies, genital infection of a murine model with *C. trachomatis* is typically less severe, requires a larger number of infection forming units, resolves faster, and produces less pathology unless directly injected transcervically into the upper genital tract (Carmichael et al., 2011; Gondek et al., 2012; De Clercq et al., 2013). Despite these characteristics, women who have a *C. trachomatis* genital infection can present with no symptoms or upper genital tract pathology. It has been argued that the clinical presentation during a genital infection in women warrants the use of *C. trachomatis* in the murine urogenital model for comparative studies to glean knowledge toward advancing human medicine (Lyons et al., 2005).

Immune responses to genital chlamydial infections are quite intricate. Different cells and mediators from both the humoral and cell-mediated components of the immune response are required for the elimination of and protection against genital chlamydial infection (Vasilevsky et al., 2014). This intracellular organism has evolved multiple methods involved in the evasion of the immune response, but in order to elucidate these mechanisms, it is important to examine the effects of the immune cells. Initial clearance is due to the activation of NK cells, macrophages, neutrophils, and mediators such as IFN-y and IL-12 (Vasilevsky et al., 2014). For protective immunity to be generated, activation and expansion of T cells via the presentation of chlamydial antigens by dendritic cells (DCs) are required (Vasilevsky et al., 2014). B cells then interact with the activated clonal T cells to produce antibodies against *Chlamydia.* The activation of B cells by helper T cells to induce T-cell dependent antibody responses occurs when both cell types interact with the same antigen. This is referred to as linked recognition in which a CD4 T cell specific for a pathogen must be activated to produce armed helper T cells before B cell activation can take place. After this occurs, B cells will produce antibodies against the specific pathogen. Even though the infection can be cleared and protective immunity is established, recurring and persisting infections in combination with a shift from Th1 to Th2 immunity appear to result in tissue damage, scarring of the reproductive tissue, and infertility (Vasilevsky et al., 2014).

During the innate immune response, neutrophils are the first to be recruited to the site of an infection, are implicated in the initial control of chlamydial infections, and are a main source of tissue damaging cytokines (Vasilevsky et al., 2014). Because they are short-lived in comparison to other leukocytes (Salamone et al., 2001), they are believed

to be responsible for the reduction of infectious progeny as well as the limitation of dissemination. Evidence has indicated that infection with *C. trachomatis* may delay apoptosis of neutrophils (Salamone et al., 2001), and this prolongation of neutrophil life-span contributes to infertility and fibrosis during chlamydial infection (Lee et al., 2010). Along with their tissue-damaging properties during infection, studies have demonstrated that human neutrophils inactivate *C. trachomatis in vitro* via the oxygen-dependent microbicidal systems of human neutrophils (Yong et al., 1982; Register et al., 1986).

Macrophages are a major component of the innate immune response that play an important role in the activation of the adaptive immune response via the presentation of antigen to T cells. *In vivo* studies have revealed that macrophages exhibit multiple roles during a chlamydial infection: production of proinflammatory cytokines (Bas et al., 2008), phagocytosing of bacteria (Beagley et al., 2009), and migration to chlamydial infection sites (Morrison and Morrison, 2000). It has also been documented that these immune cells not only provide a hostile environment for engulfed *Chlamydia*, but *C. trachomatis* destruction inside macrophages induces host cell autophagy (Al-Zeer et al., 2013). This induced autophagy enhances antigen presentation to T cells, which in turn initiates a humoral response in naïve mice (Vasilevsky et al., 2008).

In vivo and *in vitro* studies have revealed that dendritic cells (DCs), which are essential APCs, are known to secrete many cytokines which direct T cell responses during MHC class I and II antigen presentation, and aid in the mediation of the Th1/Th2 balance (Morrison et al., 1995; Su et al., 1998; Matyszak et al., 2002). Immature DCs internalize and phagocytize pathogens, break down their components, and mature to express MHC molecules and co-stimulatory molecules. Mature DCs will then migrate to

local lymph nodes and present antigen. This presentation allows T cells to activate and to initiate a cell-mediated as well as a humoral immune response. In an early study involving a nasal challenge of mice with bone marrow-derived DCs that were incubated along with heat-killed *C. trachomatis*, a protective response was established (Lu and Zhong, 1999). This response was Th1 mediated which further revealed a correlation between Th1-skewed immunity and protection against infection. For the mice that received DCs pulsed with recombinant MOMP, a Th2-associated IgG1 response was elicited instead, and this has correlated with what has been visualized in other studies (Su et al., 1998; Shaw et al., 2001; Shaw et al., 2002). There was an additional study that was performed that provided additional support in the mediation of the Th1 and Th2 balance controlled by DCs. For this study, mice were injected with *Chlamydia*-pulsed IL-10KO DC and this led to an increase in the frequency of Th1 cells (He et al., 2005). The mediation of this balance, the types of cytokines produced, and the antigens that are processed by DCs make them a critical tool for the development of vaccines.

Macrophages and DCs are critical in that both APC types activate T cells, which will migrate to the site of infection. In order for T cell activation to occur, a series of events have to take place. First, pathogen-associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PPRs). This recognition causes many events to occur that include phagocytosis/uptake and entry of engulfed material into the endocytic pathway (Vyas et al., 2008; Underhill and Goodridge, 2012). For presentation via MHC class II molecules, this material is broken down in the phagolysosome, attached to MHC molecules, and trafficked to the surface of APCs for presentation to CD4 T cells (Vyas et

al., 2008). For presentation via MHC class I molecules, peptides derived from cytosolic proteins are degraded by the proteasome. The transporter associated with antigen processing (TAP) will translocate the peptides from the cytosol into the endoplasmic reticulum, where loading onto MHC molecules occur. Finally, these complexes will be transported to cellular surfaces for display to CD8 T cells (Vyas et al., 2008). T cell involvement was shown to occur over 30 years ago when it was observed that a wild type strain of mice was able to clear a genital *C. muridarum* infection after 20 days post infection, while immunodeficient athymic nude mice established chronic infection (Rank et al., 1985). Over the years, both T cell subsets have been revealed to recognize different *C. trachomatis* antigens: major outer membrane protein (MOMP) (Holland et al., 1997; Ortiz et al., 2000; Shaw et al., 2002), chlamydial protease activating factor (CPAF) (Li et al., 2011), polymorphic outer membrane protein D (POMP-D) (Goodall et al., 2001b), outer membrane protein 2 (Omp2) (Goodall et al., 2001a), heat shock protein 60 (hsp60) (Deane et al., 1997), RpIF, PmpF, and PmpG (Olive et al., 2011; Johnson et al., 2012). Although there is copious evidence that support the role of CD4 T cells in the resolution of both *C. trachomatis* and *C. muridarum (Jayarapu et al., 2010; Gondek et al., 2012; Labuda and McSorley, 2018),* evidence for the function of CD8 T cells has been lacking. Much of the research that has been performed in mice has supported the role of CD8 cells in immunopathology and not in protection (Vlcek et al., 2016). However, there have been stipulations to these phenomena including the correlation between the identification of CD8 epitopes with resolution of infection in humans (Picard et al., 2015) and the established evidence for protection by CD8 cells with a trachoma vaccine in nonhuman primates (Olivares-Zavaleta et al., 2014). CD8 T cells are also known to destroy host

cells that are infected with *Chlamydia*, but they are not essential in the clearance of *C. trachomatis* (Su and Caldwell, 1995; Morrison and Morrison, 2000). However, they do produce IFN-y that, in turn, regulates other immune cells, such as macrophages. Due to the lack of research that either supports or rejects the role of CD8 cells in protection versus pathophysiology during infection, CD4 cells and their responses have been accepted as the more reliable protective arm of the adaptive immune response (de la Maza et al., 2017).

In order to examine the concentrations of different immune cells during any kind of infection, flow cytometry can be employed. This is a tool that illuminates particles or cells that individually flow across a source of light. Signals, which are mainly detected as antibody-antigen bound complexes from the cells/particles, are the result of the illumination. The signals are interpreted and presented in such a way that individual populations of cells/ particles can be visualized using a flow cytometry-based program. This tool has been utilized for both chlamydial *in vitro* and *in vivo* studies (Waldman et al., 1987; Olivares-Zavaleta et al., 2014; Naglak et al., 2016), but it has yet to be for a genital *C. trachomatis* murine infection model that will involve infection with a mutant that has a known function *in vitro*. For this study, a flow cytometry protocol similar to the protocol in Naglak et al. 2016 was utilized to examine the concentrations of different immune cells during genital L2-wild type and L2-ΔCT228 infection.

Hypothesis and predictions

We hypothesize that, based upon the findings in support of the previous hypothesis and the data published in Shaw et al. 2018, there will be additional

immunological differences between a L2-wild type infection and L2-ΔCT228 infection. To test this hypothesis, we will compare the recruitment of different immune cell populations to the reproductive tracts of the mice. Since infection with L2-ΔCT228 was shown to produce lower antibody titers and result in a longer duration in infected mice, we theorize that there will be a decrease in the number of different immune cell types recruited to the tracts when compared to a L2-wild type infection. To investigate this phenomenon, the goal is to harvest and digest whole, L2-wild type infected and L2-ΔCT228 infected murine reproductive tracts and analyze the concentrations of different immune cell types via flow cytometry.

Methods

Intravaginal infection of mice

Inbred, female C3H/Hej mice used for this experiment were obtained from Jackson Laboratories (Bar Harbor, Maine) at 8-10 weeks old and all were acclimated for 1 week (7 days) prior to experimentation. At -7 and -3 days before chlamydial infection, estrus was synchronized via subcutaneous injection with depo formulation of 2.5mg of medroxyprogesterone acetate (Upjohn, Kalamazoo, MI). All mice were subsequently intravaginally infected with either 1 million *C. trachomatis* L2-wild type EBs or L2-ΔCT228 EBs in 5ul SPG.

Obtaining Recoverable IFUs from the Cervicovaginal Tract

Cervicovaginal tracts were swabbed (Puritan Diagnostics, HydraFlock 6” 15 cm swabs; Guilford, ME) at day 3 and day 7 p.i. to validate infection with either L2-wild type or L2-ΔCT228. Each swab was added to microcentrifuge tubes containing both 600ul of SPG

and one glass bead the placed on ice. Each swab was vortexed to liberate EBs and serially diluted before the inoculation of confluent HeLa cell monolayers in 96 well plates (CellTreat Scientific, Pepperell MA). Entry of EBs was promoted via centrifugation for 1 hr. at 700xg. For 30min at 37°C, cells were incubated before undergoing washes to remove any extracellular EBs. For 24 hrs. at 37°C with 5% CO_2, these cells were incubate in media consisting of RPMI 1640 + 5% FBS + gentamycin (10ug/mL).The cells were fixed with methanol, stained with anti-MOMP (courtesy of Dr. Harland Caldwell), before staining with anti-mouse DyLight 488 (Jackson ImmunoResearch). Twenty fields of view were counted using a Leica MI6000B fluorescent microscope with a 20x objective to obtain the total recoverable IFUs per mouse.

Digestion and Flow Cytometry Preparation of Whole Murine Reproductive Tracts

At day 7 and day 21 p.i., three whole murine reproductive tracts (n = 9 per group) were excised, pooled, and minced with sterile scissors into RPMI medium that has been supplemented with fetal bovine serum (FBS) and with 5 mg/ml collagenase IV (Worthington Biochemical Company). All contents were transferred to 50 ml conical tubes and placed into a 37°C water bath for 20-25min. to allow activation and consistent enzymatic activity of collagenase to occur. Contents were then transferred to a digestor bag and homogenized via the Stomacher™ 400 (Seward) then transferred back to a 37°C water bath for 10-15 min. After incubation, the contents were transferred to a new digestor bag and homogenized using via the same digestor (as listed) once more. Samples were then rinsed through a 70-um cell strainer and centrifuged to pellet all cells. Fluorescence-activated cell sorter (FACS) buffer (phosphate-buffered saline (PBS) with

2% FBS) was used to rinse the pellet and Lysis buffer was used to lyse red blood cells for 2 min. while on ice. Cells were resuspended in 1 ml of FACS buffer, 100ul of cells were counted in Trypan Blue using a hemocytometer, and concentrations containing 1 million cells/mL were prepped for blocking and antibody staining. For five minutes at room temperature, Fc Block (ThermoFisher) was added to each suspension of cells before staining with the following antibodies in FACS buffer for 30min at 4°C: CD11b Monoclonal Antibody (M1/70) (ThermoFisher) Alexa Fluor 488 (ThermoFisher), CD11c Monoclonal Antibody (N418) Alexa Fluor 700 (ThermoFisher), Ly-6G(Gr-1) Monoclonal Antibody (RB6-8C5) eFluor 450 (ThermoFisher), F4/80 Monoclonal Antibody (BM8) PE-eFluor 610 (ThermoFisher), PE-Cy™7 Rat Anti-Mouse CD45 Clone 30-F11 (RUO) (BD Biosciences), CD8a Monoclonal Antibody (53-6.7) PerCP-eFluor 710 (ThermoFisher), CD4 Monoclonal Antibody (GK1.5) eFluor 660 (ThermoFisher), and Brilliant Violet 785™ anti-mouse CD3 Antibody (Biolegends). To examine live/dead cell populations, SYTOX Orange (ThermoFisher) was used per instruction of the manufacturer. Cells were washed with FACS buffer and fixed in 1 ml filter-sterilized 2% Formaldehyde in FACS buffer. Analyses of samples were completed using a NovoCyte 4000™ flow cytometer and NovoExpress™ software. The gating was determined using Compensation controls and the degree of nonspecific staining was determined by Isotype controls.

Statistics

Differences in the concentration of different immune cell types between L2-wild type and L2-ΔCT228 infected mice were determined using one-way analysis of variance (ANOVA) with the Tukey posttest. Log IFU data were analyzed at the individual time points following infection using unpaired two-tailed student t test. Statistical calculations were completed using Prism 7.0.

Results

In comparison to genital infection with L2-wild type, genital infection with L2-ΔCT228 revealed a significant difference in IFU concentration during infection at day 3 and had similar concentrations of immune cells recruited to the murine reproductive tract. Quantified, recoverable log IFUs, as shown in Figure 6A., revealed a significant decrease in IFU concentration (***p = 0.0005) in the mutant infected mice in comparison to the wild-type infected mice that was not observed at day 7 p.i. (Figure 3B). This correlated with what was observed in Shaw et al., 2018. Despite mutant infection having significantly lower IFU concentrations at day 28 p.i. and systemic antibody titers (Shaw et al. 2018), the concentrations of immune cells recruited to the murine reproductive tract were similar to what was observed during L2-wild type infection (Figure 7A-E).

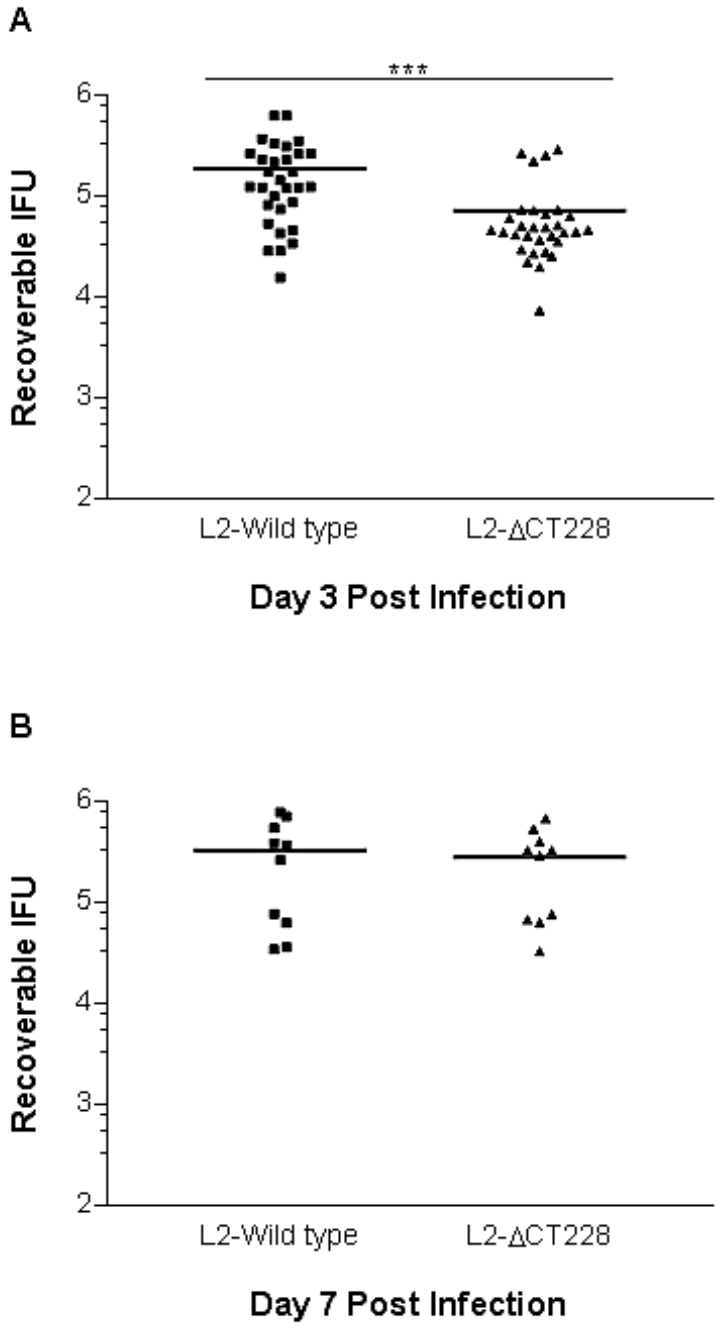
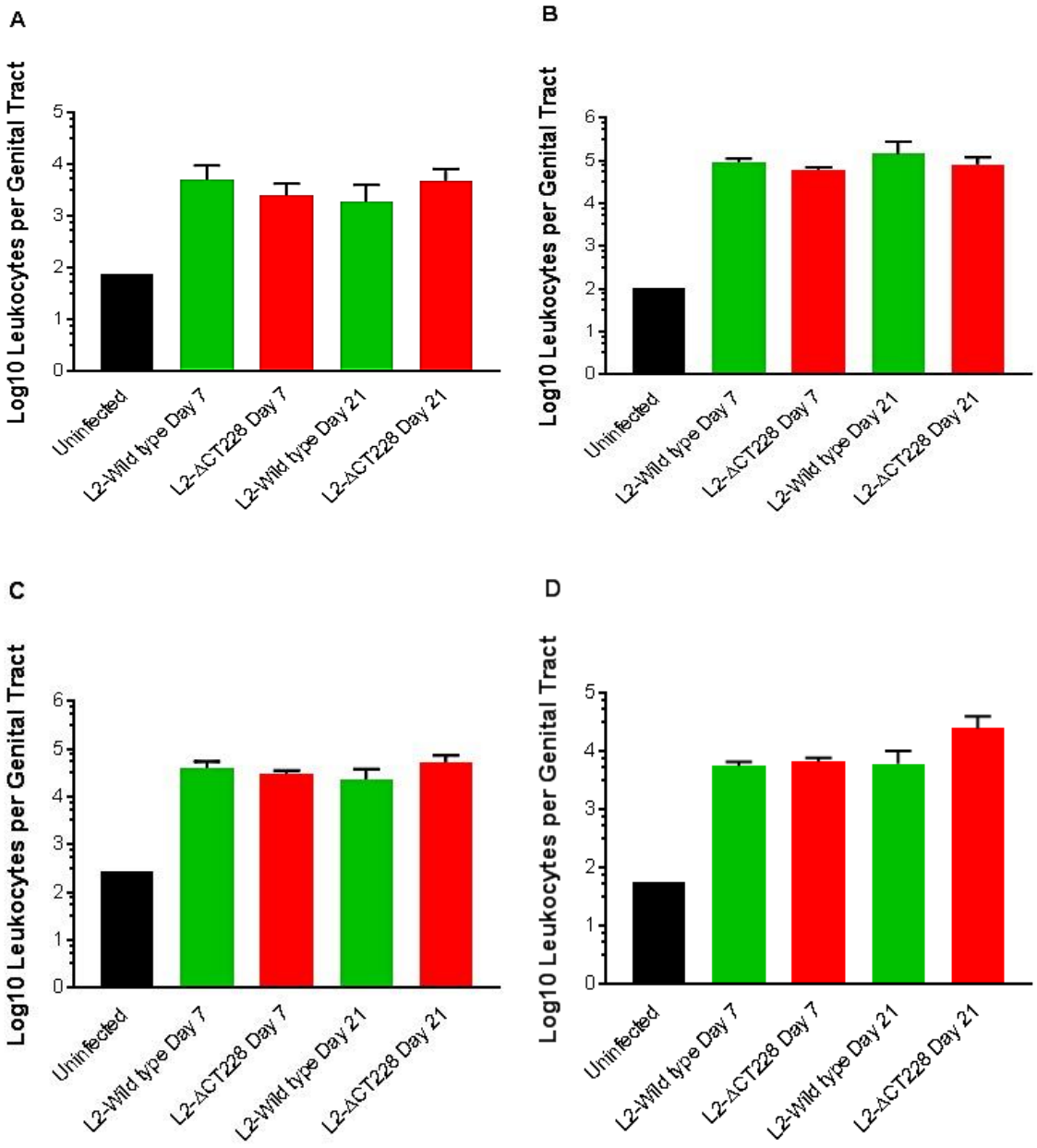


Figure 6. Significant decrease of recoverable IFUs shed by mice infected with *C. trachomatis* L2-ΔCT228 at day 3 p.i. 1×10^6 EBs of either L2-wild type or L2-ΔCT228 was administered to female C3H/HeJ mice intravaginally. Vaginal tracts were swabbed for recoverable IFUs, which were enumerated on HeLa cell monolayers. Recoverable IFU concentrations from either L2-wild type or L2-ΔCT228 infection are expressed **(A)** at day 3 p.i. (n = 31) and **(B)** at day 7 p.i. (n = 10). The data revealed a significant decrease in IFU concentrations (***$p = 0.0005$) at day 3 p.i. from the L2-ΔCT228

infected mice than what was observed from the L2-wild type infected mice. Triangles and squares represent individual mice, bars represent mean of group for each time point.



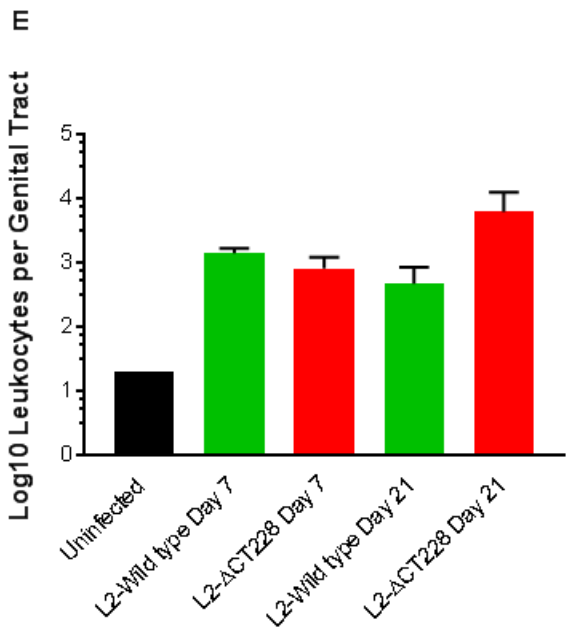


Figure 7. Recruitment of immune cells to murine, genital tracts during primary, intra-vaginal L2-wild type and L2-ΔCT228 infection. 1×10^6 EBs of either L2-wild type or L2-ΔCT228 was administered to female C3H/HeJ mice intravaginally. Three whole genital tracts (n = 9 mice/group) were harvested and pooled at day 7 and day 21 p.i. from L2-wild type and L2-ΔCT228 infected mice. Cells were isolated for flow cytometry and stained with antibodies for detection of respective immune cell populations: **neutrophils (A), macrophages (B), dendritic cells (C), CD4 T cells (D), and CD8 T cells (E).** The black bars represent the average mean of uninfected mice (n = 3 mice/group, 1 trial total), while the green and red bars represent the average mean of infected mice (n = 9 mice/group, 3 trials total). The error bars represent mean + SEM.

Discussion

From what was observed in Shaw et al., 2018, there was a significant effect of time on the L2-wild type infected mice at day 28 p.i. Most of the wild type infected mice cleared the infection in comparison to the mutant infected mice at this time point. From this result, it is speculated that more EBs were able to be cloaked from components of the immune response within the increased numbers of extrusions. However, there was no focus on events that may be taking place during the early stages of murine infection (~ day 3), and this prompted the

enumeration of IFUs at day 3. What was observed, as visualized in Fig. 6A and B, was the significant decrease in the number of mutant IFUs at day 3 in comparison to the wild type, while at day 7 p.i., there was no difference. In combination with the focus on the possibility of immune response evasion, chlamydial infectivity and internalization need to be examined further.

Chlamydial internalization has been studied extensively over the years, and this has given rise to many supported and refuted mechanisms, including clathrin-mediated endocytosis (Hodinka et al., 1988; Boleti et al., 1999) and caveola-mediated entry (Gabel et al., 2004; Webley et al., 2004) . The invasion of host cells is due to either generalized pinocytosis or directed phagocytosis, and the facilitation of internalization is due to a number of regulatory factors that may or not be critical for entry. Some of these examples include the small GTPases, Rac1 and Arf6, that have important roles (Carabeo et al., 2004; Balana et al., 2005) and the small GTPases, RhoA and Cdc42, that do not have important roles (Carabeo et al., 2004). The polymerization and rearrangement of actin also plays a role in entry as well as recruitment of the inclusion towards the MTOC (Clifton et al., 2004). Intracellular calmodulin, calcium, and calcium-activated annexins are host cellular components that have been identified to be involved in chlamydial infectivity (Murray and Ward, 1984; Majeed et al., 1994). Despite all that has been revealed, many of the cellular processes that mediate chlamydial entry and infectivity have yet to be elucidated, and this is mainly a result of the lack of hypothetical protein elucidation across chlamydial species. One important family of proteins consists of inclusion membrane proteins (Incs) that may function in

the communication between the bacteria and the host cell (Morrison et al., 2011; Elwell et al., 2016).

CT228 has been revealed to interact with MYPT1, a subunit of myosin phosphatase, and this role was examined in the context of host cell exit *in vitro (Lutter et al., 2013)*. However, at this point, there is no understanding as to what may be occurring during the initial stages of infection *in vivo* that may be due to inactivation of this protein. We have yet to know which host cell components are being utilized or which signaling pathways may be affected. In the harsh environment of an *in vivo model,* there is an overall lack of research that has been published documenting what has been visualized *in vitro* in terms of chlamydial entry and host cell manipulation. In order to be able to understand what may be occurring *in vivo*, more needs to be done *in vitro* for the elucidation of unknown proteins, such as most of the Incs, as well as their interactions with other Incs and with host cell components.

In combination with what was observed during the growth curves and enumerated IFU concentrations, mutant infection also revealed a significant decrease in systemic anti-*Chlamydia* IgG2a antibody titers (Shaw et. al. 2018). This led to the speculation that the difference in EB release, either via lysis or extrusion, could alter the degree of recognition by dendritic cells and macrophages, and this phenomenon could have led to the alteration of host immune response clearance mechanisms. To examine this further, immune cells recruited to the reproductive tracts of mice were enumerated and characterized. From the data presented in Fig. 7A-E, it was observed that there were no

statistical differences amongst the total immune cell populations per reproductive tract between L2-wild type and L2-ΔCT228 infected mice. Although whole populations were examined, it is important to note the existence of multiple cell types that have many different roles that have yet to be characterized during infection with this mutant strain: these include M1 and M2 macrophages (Mohammadi et al., 2019), iTreg, Th1 cells, Th2 cells, Th17 cells (Hirahara and Nakayama, 2016), plasmatocytoid DCs (pDCs), myeloid DCs (mDCs), CD14+ DCs, etc (Mildner and Jung, 2014).

Most of the *in vivo* research, in regards to the immune response to genital chlamydial infections, has involved infection of murine models with *Chlamydia muridarum (Vasilevsky et al., 2014).* Despite the limitations, these models have provided researchers with a basis for understanding what is involved during genital infection. It has been well established that chlamydial infection produces a robust Th1 immune response that is characterized by the secretion of IFN-y, IL-12, and TNF-α, which will lead to the activation of macrophages and CD8 T cells, as well as the production of IgG2a (Constant and Bottomly, 1997) The overall function of these components is to resolve infection, which is critical in *C. muridarum* murine infection models. When these mice are depleted of CD4 T cells that produce Th1-type cytokines, resolution does not occur. However, when these CD4 T cells are depleted in a *C. trachomatis* murine infection model, there are no effects on clearance or the development of protective immunity (Morrison et al., 2011). In contrast to what is known about chlamydial Th1 responses, Th2 responses are understudied. This response involves the secretion of IL-3, IL-4, IL-

5, IL-10, and these cytokines are important in the activation of B cells, eosinophils, and mast cells. They can also the block the production of IgG2a (Constant and Bottomly, 1997). The overall function of this response involves the maturation and differentiation of B cells. However, due to the lack of research in regards to this response, the importance in either a *C. trachomatis* or *C. muridarum* genital infection is unknown. Cytokine production in relation to immune responses has not been well characterized in a *C. trachomatis* murine infection model, and despite not observing differences in the concentrations of immune cells, the enumeration of cytokines is an essential future direction that will provide researchers information in regards to the different subtypes of immune cells that may be recruited.

Even if resolution does not occur, protective immunity is still established via the secretion of some cytokines as well as the presentation of antigen to T cells and B cells (Morrison et al., 2011). A primary infection was examined for this study, and during primary infection, minimal antibody and B cell concentrations are produced. In order to gain more of an understanding, multiple rounds of wild type and mutant infection would need to take place.

For this study, serovar L2 was used for murine infection since this is the only serovar in which an inactivated CT228 mutant has been generated. Another future direction includes the generation of CT228 mutants in other genital serovars that more closely mimic the histopathology that is observed during and after human infection. Unlike other genital serovars and *C. muridarum*, infection with serovar L2 has been documented to produce less histopathology in mice due

to many reasons: infection resolves faster, fewer IFUs are shed, higher IFUs are needed for robust infection, and no ascension into the upper genital tract occurs unless if the bacteria are injected trancervically (Morrison et al., 2011; Vasilevsky et al., 2014)

Overall, there is still much to be done in regards to the study of this mutant in a murine infection model. The main foci include the examination of the effects of decreased antigen presentation within extrusions, the enumeration and characterization of cytokines and different immune cell subtypes, the examination of host signaling pathways as infection is occurring, the establishment of protective responses, and the generation of an inactivated CT228 mutant in other serovars that more closely mimic the histopathology observed in human infection. The *in vitro* and *in vivo* characterization of more hypothetical genes will advance the understanding of *Chlamydia* as well as provide additional vaccine candidates that are, to date, desired.

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Shaw, J.H., **Key, C.E.**, Snider, T.A., Shaw, E., Fisher, D.J., and Lutter, E.L. (2018) Genetic inactivation of *Chlamydia trachomatis* inclusion membrane protein CT228 alters MYPT1 recruitment, extrusion production, and longevity of infection. Frontiers in Cellular and Infection Microbiology. DOI: 10.3389/fcimb.2018.00415

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